RESEARCH ARTICLE

High Frequency of *CDKN2A* Alterations in Esophageal Squamous Cell Carcinoma from a High-risk Chinese Population

Nan Hu,[1]† Chaoyu Wang,[1]† Hua Su,[2] Wen-Jun Li,[3] Michael R. Emmert-Buck,[1] Guang Li,[3] Mark J. Roth,[1] Ze-Zhong Tang,[3] Ning Lu,[4] Carol Giffen,[5] Paul S. Albert,[6] Philip R. Taylor,[1]* and Alisa M. Goldstein[7]*

[1]Center for Cancer Research, National Cancer Institute, Bethesda, Maryland
[2]National Vaccine and Serum Institute, Beijing, People's Republic of China
[3]Shanxi Cancer Hospital, Taiyuan, Shanxi, People's Republic of China
[4]Cancer Institute and Hospital, Chinese Academy of Medical Sciences, Beijing, People's Republic of China
[5]Information Management Service, Inc., Silver Spring, Maryland
[6]Division of Cancer Treatment and Diagnosis, National Cancer Institute, Bethesda, Maryland
[7]Division of Cancer Epidemiology and Genetics, National Cancer Institute, Bethesda, Maryland

Because previous studies have shown that loss of heterozygosity (LOH) is common on chromosome arm 9p in esophageal squamous cell carcinoma (ESCC) and that genetic alterations in *CDKN2A* and *CDKN2B* on 9p are also common, we sought to determine whether LOH and these genetic alterations are related. We performed LOH studies on chromosome bands 9p21–p22 and searched for genetic alterations of *CDKN2A* and *CDKN2B* in 56 ESCCs from a high-risk Chinese population. Seventy-three percent of patients were found to have LOH at one or more loci on chromosome bands 9p21–p22, and LOH occurred more frequently in patients with a family history of upper gastrointestinal cancer than in those with a negative family history ($P = 0.01$, global permutation test). *CDKN2A* mutations (point mutations, deletions, insertions) were observed in 25% (14 of 56) of cases, and the LOH pattern was significantly different for individuals with and without a *CDKN2A* mutation ($P = 0.01$, global test). Three new single nucleotide polymorphisms (SNPs) and 2 previously reported SNPs were identified in this group of patients. Intragenic allelic loss at polymorphic sites in *CDKN2A* was detected in 32% (18 of 56) of patients. Seven of the 56 (13%) cases exhibited what is considered classic evidence ($n = 4$) or showed potential evidence ($n = 3$) of biallelic inactivation. Only one alteration was observed in *CDKN2B*, G171A in the 5′ untranslated region. Both mutation and intragenic allelic loss in *CDKN2A* appear to play a role in the development of ESCC. ©2003 Wiley-Liss, Inc.   © 2003 Wiley-Liss, Inc.

INTRODUCTION

Loss of heterozygosity (LOH) on chromosome arm 9p is a common occurrence in most human cancers. The chromosome band 9p21–p22 region contains several tumor-suppressor genes, including *CDKN2A* and *CDKN2B*. *CDKN2A* is a cell cycle–related gene that encodes the p16 protein, which binds competitively to cyclin-dependent kinase 4 (CDK4) and thereby inhibits the interaction of CDK4 with cyclin D1 to stimulate passage through the G1 phase of the cell cycle. Inactivation of *CDKN2A* by loss (deletion) or mutation frees the cyclin-dependent kinase from inhibition, permitting constitutive phosphorylation and the consequent inactivation of *RB1*. Thus, *CDKN2A* plays an active role in *RB1*'s growth-control pathways (Sherr, 1996; Liggett, and Sidransky, 1998). *CDKN2B* encodes the p15 protein and has high structural and functional homology to *CDKN2A* and the p16 protein (Kamb et al., 1994). Multiple mechanisms of *CDKN2A* inactivation, such as point mutations, homozygous deletions, and hypermeth-

ylation, have been reported in different kinds of human cancers, including esophageal cancer (Cairns et al., 1994; Igaki et al., 1994; Mori et al., 1994; Zhou et al., 1994; Cairns et al., 1995; Liu et al., 1995; Merlo et al., 1995; Suzuki et al., 1995; Esteve et al., 1996; Sherr, 1996; Chan et al., 1997; Muzeau et al., 1997; Tanaka et al., 1997; Gamieldien et al., 1998; Xing et al., 1999a). Homozygous deletion and hypermethylation are the most frequently observed alterations inactivating *CDKN2B* in human cancers (Herman et al., 1996; Xing et al., 1999b; Jin et al., 2000).

Esophageal squamous cell carcinoma (ESCC) is one of the most common fatal cancers worldwide, and Shanxi province, a region in north-central China, has some of the highest esophageal cancer rates in the world (Li et al., 1980; National Cancer Control Office, 1980; Li, 1982). Although epidemiologic studies have indicated that tobacco smoking and alcohol consumption are the major risk factors for squamous esophageal cancer in the low-risk regions of Europe and North America, the etiologic agents in high-risk regions have yet to be convincingly identified. Within these high-risk regions, there is a strong tendency, studies have shown, toward familial aggregation, suggesting that genetic susceptibility in conjunction with potential environmental exposures may play a role in the etiology of this cancer (Li and He, 1986; Wu et al., 1989; Hu et al., 1991).

To better understand the role of genetics in the etiology of ESCC in this high-risk region and to identify potential susceptibility genes for this cancer, we conducted several studies of allelic loss in ESCC patients from Shanxi province. The results showed frequent allelic loss on all of chromosome arm 9p, suggesting that tumor-suppressor genes on this chromosome arm, such as *CDKN2A* and *CDKN2*B, may be involved in the development of ESCC (Hu et al., 1999; Hu et al., 2000). Several investigators have reported mutations and deletions of *CDKN2A* and *CDKN2B* in primary esophageal tumors and ESCC cell lines from different geographic areas, with wide variation, 0%–52%, in the *CDKN2A* mutation frequency (Cairns et al., 1994; Igaki et al., 1994; Mori et al., 1994; Zhou et al., 1994; Cairns et al., 1995; Liu et al., 1995; Merlo et al., 1995; Suzuki et al., 1995; Esteve et al., 1996; Chan et al., 1997; Muzeau et al., 1997; Tanaka et al., 1997; Gamieldien et al., 1998; Xing et al., 1999a). However, to date no study has focused on Shanxi province, a high-incidence region of China. In addition, previous allelic loss studies have not shown a correlation between LOH and *CDKN2A* or *CDKN2B* mutations or protein expression in human cancer, including ESCC (Barrett et al., 1996; Pollock et al., 2001), although this phenomenon has been observed in studies of LOH and of other tumor-suppressor genes [e.g., *BRCA2* and *RB1* in ESCC (Montesano et al., 1996; Harada et al., 1999]. In the present study, we analyzed LOH on chromosome bands 9p21–22 in 56 ESCC patients, using 4 microsatellite markers flanking *CDKN2A* and *CDKN2B*. We used single-strand conformational polymorphism (SSCP) analysis and direct sequencing to identify genetic alterations (mutations or

intragenic allelic loss) in these two genes. The relationship between LOH on the microsatellite markers and genetic alterations in *CDKN2A* and *CDKN2B* was also evaluated, and genetic alterations of *CDKN2A* and *CDKN2B* were compared with those previously identified in *TP53* and *BRCA2* to determine whether these genes are collectively inactivated.

MATERIALS AND METHODS

Patient Selection

Patients seen in 1995 and 1996 at the Shanxi Cancer Hospital in Taiyuan, Shanxi Province, People's Republic of China, who were diagnosed with ESCC and were considered candidates for curative surgical resection were identified and recruited to participate in this study. The study was approved by the institutional review boards of the Shanxi Cancer Hospital and the U.S. National Cancer Institute (NCI). A total of 56 patients with ESCC, 34 males and 22 females, were selected; all had a histologic diagnosis of esophageal squamous cell cancer confirmed by pathologists at both the Shanxi Cancer Hospital and the NCI, and histologic sections reviewed on H&E were required to contain at least 70% of the tumor. None of the patients had had prior therapy, and Shanxi was the ancestral home for all. Information on demographic and cancer lifestyle risk factors and a detailed family history of cancer were obtained as previously described (Huang et al., 2000). Fifty-six ESCC patients, of whom 34 had a family history of upper gastrointestinal (GI) cancer and 22 did not have such a family history, were evaluated. The detailed information about these ESCC patients has been described previously (Huang et al., 2000). Briefly, the mean age of the patients in this study group was 54 years, with a range of 39–65 years; 52% were smokers, 57% were drinkers, 88% ate pickled vegetables, and 75% ate calorically hot food. Seventy-seven percent of the tumors were located in the middle third of the esophagus, 39% of patients were found to have lymph node metastases, 80% of the tumors were diagnosed as grade 2, and 96% were diagnosed as stage III. Sixty-one percent of the patients reported a family history of upper GI cancer, including 24 in a first-degree relative, eight in a second-degree relative, and two in a third-degree relative. There were no significant differences between patients with and without a family history of upper GI cancer in age, gender, tumor location, tumor grade, tumor stage, lymph node metastasis, tobacco use, alcohol consumption, any
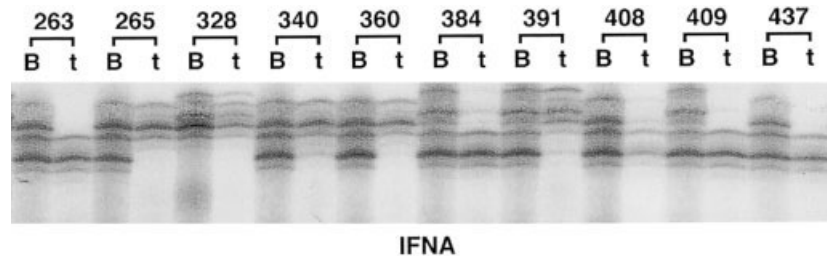
Figure 1.   LOH on IFNA in ESCC.

pickled vegetable consumption, or any calorically hot food consumption.

All patients were previously examined for *TP53* and *BRCA2* mutations: 77% (43 of 56) of patients had *TP53* somatic mutations, and 76% (22 of 29 informative cases) had intragenic allelic loss at a polymorphic site in exon 4 in their tumor (Hu et al., 2001); 9% of the patients had *BRCA2* mutations, and 46% had intragenic allelic loss at one or more polymorphic sites in their tumors (Hu et al., 2002).

Biologic Specimen Collection and Processing

Ten milliliters of venous blood was taken from each patient prior to surgery, and genomic DNA was extracted and purified. Tumor tissue obtained during surgery was fixed in ethanol and embedded in paraffin.

Laser Microdissection and Extraction of DNA

Tumor cells were obtained by laser capture microdissection using methods previously described (Hu et al., 2001).

Markers, PCR, and Microsatellite Alteration Reading and Interpretation

Four microsatellite markers, *IFNA* (GDB ID 162731), *D9S974* (GDB ID 434853), *D9S942* (GDB ID 370738), and D9S171 (GDB ID 188218), located on chromosome bands 9p21–p22, were used for testing LOH in this region. *CDKN2A* is between *IFNA* and *D9S974*, approximately 12 kb from *D9S974*, whereas *CDKN2B* is between *D9S942* and *D9S171*, approximately 22 kb from *D9S942* (Randerson-Moor et al., 2001).

DNA extracted from tumor cells microdissected from the resection specimen and genomic DNA extracted from venous blood were used for each patient. PCR reactions and LOH testing were carried out as previously described (Hu et al., 2001).

Allelic loss was defined as either complete or nearly complete loss of a band in the tumor sample relative to the corresponding normal DNA (for example, see Fig. 1). Patients whose tumor DNA

showed alleles not present in the corresponding normal DNA were classified as positive for microsatellite instability (MSI). The results were reviewed independently by three investigators (N.H., C.W., and A.M.G.).

Mutation Analysis of CDKN2A and CDKN2B Using PCR-SSCP and Direct Sequencing

All samples were screened for mutations in *CDKN2A* (exons 1α, 2, and 3) and *CDKN2B* [5′ untranslated region (UTR), exons 1 and 2] by polymerase chain reaction–single strand conformation polymorphism (PCR-SSCP) analysis. Ten pairs of primers covering intron/exon boundaries were used, including 5 pairs for *CDKN2A* (1 for exon1α, 3 for exon 2, 1 for exon 3) and 5 for *CDKN2B* (2 for 5′ UTR, 1 for exon 1, 2 for exon 2). The primer sequences are shown in Table 1. DNA and PCR reactions were the same as those used in the LOH analysis, except that PCR reactions contained 5%–10% DMSO. Typical PCR conditions were as follows: 10 min of denaturation at 94 °C; then 35 cycles at 94 °C for one min, 55–63 °C for one min, and 72 °C for one min. An elongation step at 72 °C for 10 min was added to the final cycle for all exons. The methods of SSCP and direct sequencing analysis were described previously (Hu et al., 2002). All positive results were confirmed by repetition.

Statistical Analysis

All statistical analyses were performed with Statistical Analysis Systems (SAS) (SAS Corp., Cary, NC) and S-PLUS (MathSoft, Inc., Seattle, WA) software. The difference in the pattern of tumor LOH frequency between family history–positive and –negative patients was evaluated by use of a permutation test based on the mean of the chi-square test statistics comparing individual markers (4 markers) by family history status (those with one or more first-, second-, or third-degree relatives with cancer of the esophagus, cardia, or body of the stomach were considered family history-positive). The null distribution of no difference in the pat-

TABLE I. Summary of *CDKN2A* and *CDKN2B* Primers for SSCP

Set	Exon/gene	PCR product size (bp)	Annealing temp. (C)	Name	Primers (5′–3′)
1	1/*CDKN2A*	204	60	p16-1F	gggagcagcatggagccg
				p16-1R	agtcgcccgccatcccct
2	2/*CDKN2A*	204	58	p16-2aF	agcttcctttccgtcatgc
				p16-2aR	gcagcaccaccagcgtg
3	2/*CDKN2A*	147	58	p16-2bF	agcccaactgcgccgac
				p162bR	ccaggtccacgggcaga
4	2/*CDKN2A*	189	55	p16-2cF	tggacgtgcgcgatgc
				p162cR	ggaagctctcagggtacaaattc
5	3/*CDKN2A*	169	60	p16-3F	cggtagggacggcaagagag
				p16-3R	cctgtaggaccttcggtgactga
6	5′UTR/*CDKN2B*	212	60	p15-1aF	ccagagcgaggcggggca
				p15-1aR	cctcccgtcgtccttctg
7	5′UTR/*CDKN2B*	240	60	p15-1bF	agcgccaggaaaagcccg
				p15-1bR	atgcccttgttctcctcg
8	1/*CDKN2B*	272	58	p15-1cF	cgtctgggggctgcggaa
				p15-1cR	tacaaatctacatcggcg
9	2/*CDKN2B*	221	58	p15-2aF	tgaccactctgctctctc
				p15-2aR	tccacgggcagacgacc
10	2/*CDKN2B*	209	58	p15-2bF	tgccactctcacccgacc
				p152bR	tgggcggctggggaacct

tern of LOH frequency across family history groups was obtained by randomly permuting family history status 5,000 times and evaluating the distribution of the mean chi-square value. The two-sided P value was estimated as the proportion of permuted mean chi-square values that were larger (in absolute value) than the mean chi-square value observed for the actual data. The permutation test provides a global test of the difference in the pattern of LOH frequency across family history status, thereby avoiding the inherent problem of multiple comparisons when testing differences at each marker location.

In an identical way, we used a global permutation test to compare the pattern of LOH frequency by *CDKN2A*, *BRCA2*, and *TP53* status. When the global tests were significant, we used Fisher's exact test to assess the association between individual LOH frequency and family history and *CDKN2A*, *BRCA2*, or *TP53* status. We also used Fisher's exact test to evaluate the relationship between genetic alterations of *CDKN2A*, *CDKN2B*, *TP53*, and *BRCA2* along with the association between family history and genetic alternations. All P values were two-sided and considered statistically significant at $P < 0.05$.

RESULTS

Microsatellite Alterations (LOH and MSI) of Chromosome Bands 9p21–p22 in ESCC

Microsatellite alteration analysis of the 56 ESCC patients showed that 38 (73%) had LOH at one or

more of the four loci tested, 14 (27%) were informative but had no LOH at one or more of the four markers, and 4 were homozygous for all markers. The frequencies of LOH for individual microsatellite markers *IFNA*, *D9S974*, *D9S942*, and *D9S171* were 90% (26 of 29 informative cases), 67% (18 of 27 informative cases), 52% (22 of 42 informative cases), and 55% (6 of 11 informative cases), respectively (Table 2; Fig. 1 for example of LOH in *IFNA*).

The pattern of LOH frequency was different in cases with a family history of upper GI cancer compared to cases with no family history ($P = 0.01$, global permutation test). This result was primarily due to the higher frequency of LOH at marker *D9S974* in family history–positive versus family history–negative cases (14 of 16, or 88%, versus 4 of 11, or 36%, $P = 0.01$). No significant differences were seen in LOH frequency by family history for *D9S942* (64% versus 35%, $P = 0.11$), *IFNA* (89% versus 90%, $P = 1.00$), or *D9S171* (50% versus 60%, $P = 1.00$). The frequency of LOH at these four markers was not significantly associated with other clinical characteristics or cancer lifestyle risk factors.

Evidence for MSI was observed twice with *D9S974*, twice with *D9S942*, and once with *D9S171*. Case 328 showed MSI positivity at both *D9S974* and *D9S171* (Table 2). A higher frequency of MSI was found in patients with a family history of upper GI cancer than those without a family

history (3 of 34, or 9%, versus 1 of 22, or 5%, respectively), but the difference was not significant (P = 1.00, Fisher's exact test). Overall, only 7% (4 of 56) of the cases were found to have evidence of MSI, suggesting that MSI occurs infrequently within chromosomal region 9p21–p22 in ESCC.

Mutations of *CDKN2A* in ESCC

The coding regions of *CDKN2A* were screened with PCR-SSCP, followed by direct sequencing of samples with aberrant band shifts. The results showed that 14 of the 56 cases (25%) had mutations, of which five were deletions, three were insertions, and seven were point mutations (Table 3 and Figs. 2 and 3). All five deletions were found in exon 1 and ranged from 2 to 18 bp; two cases (021 and 459) lost the same six amino acids. Two insertions (cases 150 and 308) and one deletion (case 252) resulted in frame shifts and subsequent stop codons. The seven point mutations included one nonsense mutation, one missense mutation, one point mutation followed by a 1-bp insertion (case 150), two silent mutations, and two point mutations in introns, of which one was at a splice donor site (Table 3 and Figs. 2 and 3). Taken together, truncating alterations were found in six cases (11%; cases 252, 360, 150, 308, 098, and 069; Table 3), and alterations with unknown significance were found in eight cases (14%). In addition, four cases (252, 459, 150, and 308) had both a mutation and intragenic allelic loss of the wild-type allele in the same exon, suggesting that these patients received two hits. Finally, a single case (516) was found to contain a silent germ-line mutation and loss of the mutated allele (Table 3).

Intragenic Allelic Loss of *CDKN2A* in ESCC

We identified five single nucleotide polymorphisms (SNPs), including three at nearly consecutive bases in exon 2, P81P (CCC→CCG), H83H (CAC→CAT), and D84D (GAC→GAT), none of which have previously been reported. The other two SNPs we identified, A148T and C540G (in the 3 UTRs), have been reported previously (Kumar et al., 1998; Randerson-Moor et al., 2001). Table 4 shows the frequency of genotypes and intragenic allelic loss at these five SNPs in *CDKN2A*. Overall, 18 cases (32%) had intragenic allelic loss at one or more polymorphic sites, including 17 patients with allelic loss of three nearly consecutive SNPs in exon 2, and a single case (069) that had allelic loss at two separate SNPs, one in exon 1 and the other in exon 3. In addition, three cases (069, 360, and 409)

had a mutation and intragenic allelic loss in different exons, consistent with biallelic inactivation.

Mutations of *CDKN2B* in ESCC

We screened all coding regions and the 5′ UTR of *CDKN2B* by using PCR-SSCP and direct sequencing to identify mutations in this group of patients. Only one case (093) with a positive family history of upper GI cancer had a mutation (G→A, G171A in the 5′ UTR). Given the lack of alterations in *CDKN2B*, no further associations were assessed.

Association Between LOH on Chromosome Bands 9p21–p22 and Genetic Alterations of *CDKN2A*

The pattern of LOH frequency was significantly different between patients with and without a *CDKN2A* alteration (global test, P = 0.01). Although the LOH frequency was higher on all four markers for patients with a *CDKN2A* alteration than with patients without an alteration, only one marker (*D9S974*) showed a significantly different frequency (87% versus 42%, Fisher's exact test, P = 0.04).

Association Between LOH on Chromosome Bands 9p21–p22 and Genetic Alterations of *BRCA2* and *TP53*

The pattern of LOH frequency was not significantly different by *BRCA2* genetic alteration (global test, P = 0.50). Similarly, LOH frequency was not different by intragenic allelic loss of *BRCA2* and *TP53* (global tests, P = 0.43 and 0.89, respectively). There were too few cases without genetic alterations on *TP53* to enable us to conduct this analysis.

Association Between Genetic Alterations of *CDKN2A* and *BRCA2, TP53*

We compared genetic alterations (including mutations and intragenic allelic loss) between *CDKN2A*, *BRCA2*, and *TP53*, but found no association between *CDKN2A* and *BRCA2* (13 with alterations in both genes, 18 with alterations only in *BRCA2*, 15 with alteration in *CDKN2A* only, 10 with no alterations in either gene; P = 0.28) or with *TP53* (26 with alterations in both genes, 24 with alterations only in *TP53*, two with alterations only in *CDKN2A*, four with no alterations in either gene; P = 1.00). However, the power for these assessments may have been limited.

DISCUSSION

Mutations in *CDKN2A* have been detected in esophageal cancer with frequencies varying from

TABLE 2. LOH on Chromosome Arm 9p and Genetic Alterations* in CDKN2A

No.	Patient ID	Family history of upper gastrointestinal cancer	Mutation/intragenic allelic loss		LOH	CDKN2A		LOH		
			TP53	BRCA2	IFNA	Mutation (exon)	Intragenic allelic loss (exon)	9S974	9S942	9S171
1	SHE252	5 EC (father, mother, 2 sisters, paternal grandmother)	Y/Y	N/N	L	Y(1)	Y(1, 2)	L	NI	NI
2	SHE360	EC (father), BC (brother)	N/Y	Y/Y	L	Y(1)	Y(2)	L	NI	L
3	SHE409	EC (father)	N/N	N/Y	L	Y(1)	Y(2)	L	L	NI
4	SHE021	2 EC (father, paternal grandfather)	N/Y	N/N	L	Y(1)	N	NI	L	NI
5	SHE516	2 EC (mother, brother)	Y/Y	N/N	NI	Y(1)	Y(1)	NI	L	L
6	SHE200	N	N/Y	N/N	NI	Y(1)	N	L	R	R
7	SHE459	EC (paternal aunt)	Y/N	N/Y	NA	Y(1)	Y(1)	L	L	NI
8	SHE069	N	Y/N	N/N	L	Y(2)	Y(1, 3)	L	L	L
9	SHE150	EC (mother)	N/N	Y/N	NI	Y(2)	Y(2)	L	L	NI
10	SHE308	N	Y/N	N/N	NI	Y(2)	Y(2)	L	L	L
11	SHE098	EC (paternal grandmother)	N/Y	N/N	NI	Y(2)	N	NI	NI	NI
12	SHE093	EC (father)	N/Y	N/N	NI	Y(2)	N	NI	R	R
13	SHE138	EC (mother)	Y/N	Y/Y	NI	Y(2)	N	NI msi	R	R
14	SHE322	N	Y/Y	N/N	NI	Y(2)	N	NI	L	NI
15	SHE152	CC (mother)	Y/N	N/N	L	N	Y(2)	L	L	NI
16	SHE480	EC (paternal uncle)	Y/Y	N/Y	NA	N	Y(2)	NA	L	NI
17	SHE495	EC (father)	Y/N	N/Y	L	N	Y(2)	L	L	L
18	SHE437	2 EC (father, mother)	Y/N	Y/N	L	N	Y(2)	L	R	R
19	SHE340	CC (father), CC (paternal uncle)	Y/Y	N/N	NI	N	Y(2)	L	R	R
20	SHE198	N	Y/Y	N/Y	L	N	Y(2)	L	L	NI
21	SHE052	N	Y/N	N/N	L	N	Y(2)	NI	NA	NI
22	SHE240	N	Y/N	N/N	NI	N	Y(2)	NI	L	NI
23	SHE384	2 EC (father, sister), BC (brother)	Y/N	N/Y	NI	N	Y(2)	R	NI	NI
24	SHE096	N	Y/Y	N/Y	NI	N	Y(2)	R	NI	R
25	SHE118	N	Y/N	N/Y	L	N	Y(2)	R	R	NI
26	SHE263	3 EC (maternal uncle, maternal grandfather, maternal grandmother)	Y/Y	N/Y	NI	N	Y(2)	NA	L	NI
27	SHE216	N	Y/Y	N/N	L	N	Y(2)	NI	R	NI
28	SHE497	N	Y/N	N/N	NI	N	Y(2)	NI	NI msi	NI
29	SHE108	EC (paternal uncle), CC (paternal uncle)	N/N	N/Y	L	N	N	L	L	NI
30	SHE408	EC (paternal uncle's son)	Y/N	N/Y	L	N	N	L	L	NI
31	SHE186	EC (paternall uncle), CC (brother)	Y/N	N/Y	L	N	N	L	NA	NI
32	SHE066	EC (mother), CC (father)	Y/Y	N/N	NI	N	N	L	R	NI
33	SHE444	EC (father)	Y/Y	N/Y	L	N	N	L	L	NI
34	SHE265	EC (father), BC (mother)	Y/N	N/N	L	N	N	NI	NI	NI
35	SHE235	EC (father)	Y/Y	N/Y	L	N	N	NI	NI	NI
36	SHE123	2 EC (paternal uncle, paternal grandfather)	Y/N	N/Y	NI	N	N	NI	L	NI

(Continued)

TABLE 2. LOH on Chromosome Arm 9p and Genetic Alterations* in CDKN2A (Continued)

No.	Patient ID	Family history of upper gastrointestinal cancer	Mutation/intragenic allelic loss		LOH	CDKN2A		LOH		
			TP53	BRCA2	IFNA	Mutation (exon)	Intragenic allelic loss (exon)	9S974	9S942	9S171
37	SHE095	N	Y/N	N/Y	L	N	N	NI	L	NI
38	SHE391	EC (father)	N/Y	N/N	L	N	N	R	L	NI
39	SHE273	N	N/N	N/Y	L	N	N	R	R	NI
40	SHE109	EC (paternal uncle)	Y/N	N/Y	L	N	N	NA	R	NI
41	SHE034	N	Y/N	N/N	L	N	N	R	R	NI
42	SHE247	N	Y/N	Y/N	L	N	N	R	NI	NI
43	SHE297	N	Y/N	N/N	L	N	N	R	R	NI
44	SHE328	2 EC (paternal uncle, paternal cousin)	Y/N	N/Y	R	N	N	NI	L msi	NI msi
45	SHE080	EC (father)	Y/N	N/Y	R	N	N	NI msi	R	R
46	SHE027	CC (brother)	Y/N	N/Y	NA	N	N	R	R	NI
47	SHE081	EC (mother)	Y/N	N/N	NI	N	N	NI	R	NI
48	SHE170	N	N/N	N/Y	R	N	N	NI	R	NI
49	SHE208	N	N/Y	N/N	NI	N	N	NI	R	NI
50	SHE261	N	Y/N	N/N	NI	N	N	NI	R	NI
51	SHE488	N	Y/N	N/Y	NI	N	N	R	NI	NI
52	SHE507	N	Y/Y	N/N	NI	N	N	NI	R	NI
53	SHE510	N	Y/N	N/Y	NI	N	N	NI	R	NI
54	SHE057	EC (sister)	Y/N	N/Y	NI	N	N	NI	NI	NI
55	SHE083	EC (father)	Y/Y	N/Y	NI	N	N	NI	NI	NI
56	SHE113	EC (maternal uncle)	N/N	N/N	NI	N	N	NI	NI	NI
		Frequency	91% (51/56)	55% (31/56)	90% (26/29)	25% (14/56)	32% (18/56)	67% (18/27)	52% (22/42)	55% (6/11)

*Mutations detected in TP53, BRCA2, and CDKN2A were heterozygous.
LOH: L, loss; R, retention; NI, not informative
msi, microsatellite instability
NA, data not available
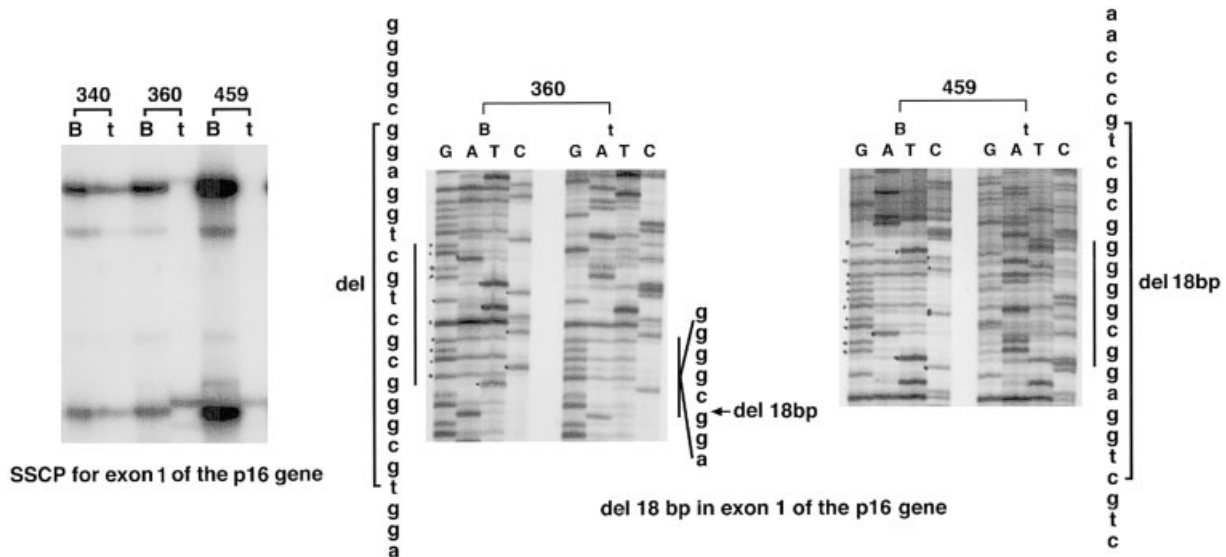EC, esophageal cancer; CC, cardia cancer; BC, body of stomach cancer.

Figure 2. (a) SSCP and (b) sequence for mutation in exon 1 of *CDKN2A*.

0% to 52% (Cairns et al., 1994; Igaki et al., 1994; Mori et al., 1994; Okamoto et al., 1994; Zhou et al., 1994; Cairns et al., 1995; Liu et al., 1995; Merlo et al., 1995; Suzuki et al., 1995; Esteve et al., 1996; Chan et al., 1997; Muzeau et al., 1997; Tanaka et al., 1997; Gamieldien et al., 1998; Xing et al., 1999a). Most of these studies examined few patients/tumors, and differences in ethnicity and geography may have been influential. For example, the mutation frequencies reported in Chinese patients were highly variable. In our study, we observed *CDKN2A* mutations in 25% of cases and intragenic allelic loss in 32%. In Hong Kong Chinese, mutations were found in only 12% (3 of 25) of patients (Chan et al., 1997), whereas in Linxian, in northern China,which also has a high-risk population, no mutations were found in 34 patients, although 17% were reported to have homozygous deletions (Xing et al., 1999a; Xing et al., 1999b). Differences in experimental methods between studies almost certainly affected mutation detection rates. It is likely that several of the methods we used (i.e., laser microdissection, small PCR products, and low-wattage/long-duration gel runs) contributed to our higher rate of detection of genetic alterations.

Intragenic allelic loss in the *CDKN2A* gene was found in 32% of ESCC patients in our study. To our knowledge, this is the first report of intragenic allelic loss at polymorphic sites in the *CDKN2A* gene in any cancer, including ESCC. Although we have no evidence for this, intragenic allelic loss could potentially cause reduced gene expression

through mechanisms such as haploinsufficiency. The importance of intragenic allelic loss is further illustrated by the fact that a microsatellite marker may point only to a region containing a tumor-suppressor gene, whereas intragenic allelic loss represents a genetic change inside a gene, and should be the functional equivalent of a deletion within that gene. Although the exact consequences of such alterations remain unclear, alterations inside the gene (i.e., intragenic allelic loss) may more directly identify genes playing important roles in tumor development. Thus, our finding of intragenic loss in 30% (17 of 56) of our patients that spanned three nearly consecutive SNPs (P81P, H83H, D84D) may be caused by somatic recombination or nonreciprocal translocation, or a loss of 12 bp (presumably codon 82 was also deleted), and this finding is more likely to represent a region of potential importance in ESCC carcinogenesis. These three nearly consecutive SNPs should be examined in future case–control studies to better understand their association with ESCC.

From the combined mutation and intragenic allelic loss results in our study, we also identified four cases with evidence of classic two-hit changes (Table 3), along with three additional cases (SHE069, SHE360, and SHE409 had a mutation and intragenic allelic loss in different exons, Table 2) that the evidence shows also may have two hits, suggesting that biallelic inactivation of *CDKN2A* does occur in ESCC. Because we lacked sufficient DNA, we did not evaluate promoter region methylation here, although this epigenetic event is
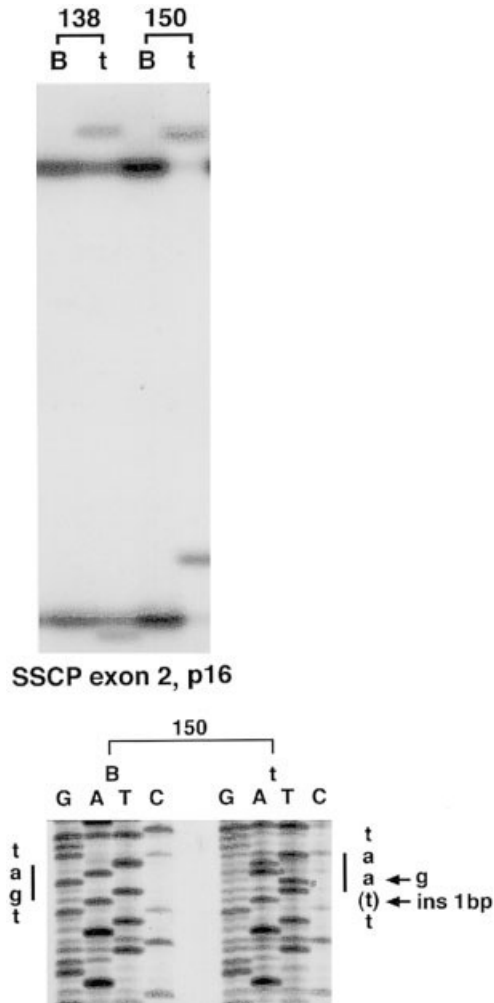
Figure 3.　(a) SSCP and (b) sequence for mutation in exon 2 of *CDKN2A*.

known to be a common occurrence with *CDKN2A* (Merlo et al., 1995; Xing et al., 1999a). As a result, we almost certainly underestimated the true frequency of biallelic inactivation in our cases. Whereas our data are consistent with *CDKN2A*'s role as a tumor-suppressor gene, confirmation of biallelic inactivation may necessitate quantitative analysis of *CDKN2A* mRNA (unavailable for the patients studied here), protein analysis, and animal or other model systems.

Homozygous deletion (HD) of both *CDKN2A* and *CDKN2B* has been reported frequently in other cancers (Jen et al., 1994) and in ESCC cell lines (Igaki et al., 1994; Tanaka et al., 1997), but infrequently in primary ESCC (Liu et al., 1995; Esteve et al., 1996; Chan et al., 1997; Xing et al., 1999a). The best method for evaluating HD is Southern blot analysis, which detects HD of an entire gene.

Unfortunately, we did not have sufficient material to conduct Southern blot analysis for either of these genes. Our mutational analysis of *CDKN2A* identified 14 mutations that were generally dispersed throughout the gene. However, two of the cases contained a mutation in three similar but distinct regions. These included mutations in codon 28 (two cases with deletion), in codons 32–37 (two cases with identical 18-bp deletions), which have been reported previously in patients from France (Muzeau et al., 1997), and in codon 79 (one insertion, one silent mutation). Each of these three codon regions merits further examination in other studies of ESCC to determine whether these findings reflect potential mutational hot spots.

Hypermethylation in the promoter regions of *CDKN2A* and *CDKN2B* has been reported to be associated with loss of protein expression in ESCC (Xing et al., 1999b; Kempster et al., 2000; Hibi et al., 2001), and these results suggested that methylation plays an important role in carcinogenesis. For an optimal understanding of the consequences of genetic alterations, both mutation and methylation of *CDKN2A* and *CDKN2B* should be examined at the same time. Unfortunately, methylation could not be examined in our study because tissues from the patients examined here were used up from numerous earlier evaluations. Our previous examination of *TP53* and *BRCA2* in these patients, however, permitted us to evaluate *CDKN2A* in conjunction with these genes simultaneously. We found that nearly half our patients ($n = 26$) had alterations in both *CDKN2A* and *TP53* [e.g., SHE252 had a 2-bp deletion in *CDKN2A* and a nonsense mutation in *TP53* (Hu et al., 2001)]. We also found that 23% ($n = 13$) of this group of ESCC patients had alterations in both *CDKN2A* and *BRCA2* [e.g., SHE360 carried a frame-shift mutation of *CDKN2A* and a missense mutation of *BRCA2* (Hu et al., 2002)]. Gamieldien et al. previously reported that three ESCC patients from a high-incidence area in South Africa carried mutations in both *CDKN2A* and *TP53* (Gamieldien et al., 1998), but there have been no reports in the literature to date of ESCC patients with both *CDKN2A* and *BRCA2* mutations. Taken together, these results indicate that numerous alterations in multiple tumor-suppressor genes occur in ESCC patients from high-risk areas, including both China and South Africa, and that more study is needed for full elucidation of the precise role of these tumor-suppressor genes in the multistep carcinogenesis process of ESCC.

A high frequency of allelic loss at chromosome bands 9p21–p22 was found in ESCC from this

TABLE 3. Mutations* in CDKN2A and CDKN2B in ESCC

No.	Patient ID	Exon/gene	Codon	Nucleotide change	Amino acid change[b]	Designation	Allelic loss at the mutation site in tumor
1	SHE252[a]	I/CDKN2A	28	del 2bp, gagg (tg)^cggg	stop codon 42	frameshift 28–41 codons	lost wild-type allele
2	SHE360[a]	I/CDKN2A	28	del 18bp, gagg (tgcggcgctgcctggagg) cggggg		frame shift from codon 28	No
3	SHE516	I/CDKN2A	29	C→A (CGG→AGG)	Arg→Arg	silent mutation in germ-line DNA	lost mutated allele
4	SHE021	I/CDKN2A	32–37	del 18 bp, gcgctg (ctggaggcggggcgctg)cccaac		lost 6 amino acids (L, E, A, G, A, L)	No
5	SHE459	I/CDKN2A	32–37	del 18bp, gcgctg (ctggaggcggggcgctg)cccaac		lost 6 amino acids (L, E, A, G, A, L)	lost wild-type allele
6	SHE200	I/CDKN2A	44–45	del 3bp, agtta (cgg) tcgg		lost 1 amino acid, glycine (next to splice site)	No
7	SHE150[a]	2/CDKN2A	52	1) between NT 155–156, ins 1 bp, gtcat (t)gatga 2) at NT156 G→A		reading frame shift resulting in a stop codon at codon 119	lost wild-type allele
8	SHE093	2/CDKN2A	65	T→C (CTC→CCC)	Leu→Pro	missense mutation	No
9	SHE138	2/CDKN2A	79	C→G (ACC→ACG)	Thr→Thr	silent mutation	No
10	SHE308[a]	2/CDKN2A	79	ins 1 bp, ctc (a)acc		reading frame shift resulting in a stop codon at codon 119	lost wild-type allele
11	SHE098[a]	2/CDKN2A	80	C→T (CGA→TGA)	Arg→stop	nonsense mutation	No
12	SHE069[a]	intron I/CDKN2A		tggca [G→A] (exon1 gtcat)		at splice donor site (AG)	No
13	SHE409	5'UTR/CDKN2A		Ins 4 bp, ggagca (agca)gc[exon1-atggagc]			No
14	SHE322	intron I/CDKN2B		gtt [C→G]tct			No
15	SHE093	5'UTR/CDKN2B	NT171	G→A			No

*Mutations detected in CDKN2A and CDKN2B were heterozygous ones.

[a]Truncating alterations.

[b]Amino acid changes in p14^ARF: #7, Asp67 stop; #8, Ala79Ala; #9, Pro94Ala; #10, frame shift 93–145 stop 145; #11, Pro94Leu.

TABLE 4. Single Nucleotide Polymorphisms (SNPs) and Intragenic Allelic Loss in *CDKN2A* in ESCC

SNP	Exon	Nucleotide change	Amino acid change	Genotype (frequency)		
				Homozygous wild-type	Heterozygous (allelic loss in tumor[a])	Homozygous variant
P81P	2	CCC→CCG	Pro→Pro	0 (0%)	44 (79%) (LW = 6, LV = 11)	12 (21%)
H83H	2	CAC→CAT	His→His	0 (0%)	44 (79%) (LW = 6, LV = 11)	12 (21%)
D84D	2	GAC→GAT	Asp→Asp	0 (0%)	44 (79%) (LW = 6, LV = 11)	12 (21%)
A148T	2	GCG→ACG	Ala→Thr	52[b] (98%)	1 (2%) (LW = 1)	0 (0%)
C540G	3′UTR	C→G	—	51 (91)	5 (9%) (LW = 1, LV = 0)	0 (0%)

[a]LW, lost wild-type allele; LV, lost variant allele.
[b]3 cases failed PCR.

high-risk Chinese population. Marker *IFNA* showed the highest LOH frequency; however, LOH at this marker was not significantly associated with genetic alterations in either *CDKN2A* or *CDKN2B*, suggesting that it may be related to other nearby genes. *D9S974* is the marker closest to *CDKN2A*, and *D9S942* is closest to *CDKN2B* (Randerson-Moor et al., 2001). Both of these markers showed higher frequencies of LOH in patients with a family history of upper GI cancer than in patients with no family history, but the difference was significant only for *D9S974*. Whereas LOH at *D9S974* was significantly associated with the presence of a genetic alteration in *CDKN2A*, the number of cases evaluated here was small, and it remains for future studies to determine the meaning of this association.

Our results show that higher frequencies of LOH, MSI, and mutations of *CDKN2A* and *CDKN2B* were found in patients with a family history of upper GI cancer than in patients with no family history, but the differences were not significant except for LOH at *D9S974*. This suggests that patients with a positive family history may have a higher degree of chromosomal instability (LOH and MSI) and that these alterations may be involved in changes occurring in nearby genes (*CDKN2A*) that may be involved in the development of ESCC.

In summary, genetic alterations on chromosome arm 9p were common in this group of ESCC patients. Seventy-three percent of patients were found to have LOH at one or more loci at chromosome bands 9p21–p22. Mutations in *CDKN2A* were detected in 25% of patients and included deletions, insertions, and point mutations. Three new and two previously reported SNPs were identified. Intragenic allelic loss at these polymorphic sites in *CDKN2A* was detected in 32% of patients. When mutations and intragenic allelic loss were consid-

ered together, evidence for classic biallelic inactivation was present for four patients, whereas three additional cases showed potential biallelic inactivation. Only one mutation was observed in *CDKN2B*. Although HD is the most frequently reported alteration in *CDKN2B*, we did not have sufficient material to examine this. Taken together, these results indicate that genetic alterations in *CDKN2A* are extremely common in ESCC and that, most likely, these changes at least partly explain the high rates of LOH seen on chromosome arm 9p. In contrast, mutations in *CDKN2B* are extremely rare in primary ESCC. Further studies will be needed for an examination of HD.

REFERENCES

Barrett MT, Sanchez CA, Galipeau PC, Neshat K, Emond M, Reid BJ. 1996. Allelic loss of 9p21 and mutation of the CDKN2/p16 gene develop as early lesions during neoplastic progression in Barrett's esophagus. Oncogene 13:1867–1873.

Cairns P, Mao L, Merlo A, Lee DJ, Schwab D, Eby Y, Tokino K, van der RP, Blaugrund JE, Sidransky D. 1994. Rates of p16 (MTS1) mutations in primary tumors with 9p loss. Science 265:415–417.

Cairns P, Polascik TJ, Eby Y, Tokino K, Califano J, Merlo A, Mao L, Herath J, Jenkins R, Westra W. 1995. Frequency of homozygous deletion at p16/CDKN2 in primary human tumours. Nat Genet 11:210–212.

Chan WC, Tang CM, Lau KW, Lung ML. 1997. p16 Tumor suppressor gene mutations in Chinese esophageal carcinomas in Hong Kong. Cancer Lett 115:201–206.

Esteve A, Martel-Planche G, Sylla BS, Hollstein M, Hainaut P, Montesano R. 1996. Low frequency of p16/CDKN2 gene mutations in esophageal carcinomas. Int J Cancer 66:301–304.

Gamieldien W, Victor TC, Mugwanya D, Stepien A, Gelderblom WC, Marasas WF, Geiger DH, van Helden PD. 1998. p53 and p16/CDKN2 gene mutations in esophageal tumors from a high-incidence area in South Africa. Int J Cancer 78:544–549.

Harada H, Tanaka H, Shimada Y, Shinoda M, Imamura M, Ishizaki K. 1999. Lymph node metastasis is associated with allelic loss on chromosome 13q12–13 in esophageal squamous cell carcinoma. Cancer Res 59:3724–3729.

Herman JG, Jen J, Merlo A, Baylin SB. 1996. Hypermethylation-associated inactivation indicates a tumor suppressor role for p15INK4B. Cancer Res 56:722–727.

Hibi K, Taguchi M, Nakayama H, Takase T, Kasai Y, Ito K, Akiyama S, Nakao A. 2001. Molecular detection of p16 promoter methylation in the serum of patients with esophageal squamous cell carcinoma. Clin Cancer Res 7:3135–3138.

Hu N, Dawsey SM, Wu M, Taylor PR. 1991. Family history of oesophageal cancer in Shanxi Province, China. Eur J Cancer 27:1336.

Hu N, Roth MJ, Emmert-Buck MR, Tang ZZ, Polymeropolous M, Wang QH, Goldstein AM, Han XY, Dawsey SM, Ding T, Giffen C, Taylor PR. 1999. Allelic loss in esophageal squamous cell carcinoma patients with and without family history of upper gastrointestinal tract cancer. Clin Cancer Res 5:3476–3482.

Hu N, Roth MJ, Polymeropolous M, Tang ZZ, Emmert-Buck MR, Wang QH, Goldstein AM, Feng SS, Dawsey SM, Ding T, Zhuang ZP, Han XY, Ried T, Giffen C, Taylor PR. 2000. Identification of novel regions of allelic loss from a genomewide scan of esophageal squamous-cell carcinoma in a high-risk Chinese population. Genes Chromosomes Cancer 27:217–228.

Hu N, Huang J, Emmert-Buck MR, Tang ZZ, Roth MJ, Wang C, Dawsey SM, Li G, Li WJ, Wang QH, Han XY, Ding T, Giffen C, Goldstein AM, Taylor PR. 2001. Frequent inactivation of the TP53 gene in esophageal squamous cell carcinoma from a high-risk population in China. Clin Cancer Res 7:883–891.

Hu N, Li G, Li WJ, Wang C, Goldstein AM, Tang ZZ, Roth MJ, Dawsey SM, Huang J, Wang QH, Ding T, Giffen C, Taylor PR, Emmert-Buck MR. 2002. Infrequent mutation in the BRCA2 gene in esophageal squamous cell carcinoma. Clin Cancer Res 8:1121–1126.

Huang J, Hu N, Goldstein AM, Emmert-Buck MR, Tang ZZ, Roth MJ, Wang QH, Dawsey SM, Han XY, Ding T, Li G, Giffen C, Taylor PR. 2000. High frequency allelic loss on chromosome 17p13.3–p11.1 in esophageal squamous cell carcinomas from a high incidence area in northern China. Carcinogenesis 21:2019–2026.

Igaki H, Sasaki H, Kishi T, Sakamoto H, Tachimori Y, Kato H, Watanabe H, Sugimura T, Terada M. 1994. Highly frequent homozygous deletion of the p16 gene in esophageal cancer cell lines. Biochem Biophys Res Commun 203:1090–1095.

Jen J, Harper JW, Bigner SH, Bigner DD, Papadopoulos N, Markowitz S, Willson JK, Kinzler KW, Vogelstein B. 1994. Deletion of p16 and p15 genes in brain tumors. Cancer Res 54:6353–6358.

Jin M, Piao Z, Kim NG, Park C, Shin EC, Park JH, Jung HJ, Kim CG, Kim H. 2000. p16 is a major inactivation target in hepatocellular carcinoma. Cancer 89:60–68.

Kamb A, Gruis NA, Weaver-Feldhaus J, Liu Q, Harshman K, Tavtigian SV, Stockert E, Day RS, III, Johnson BE, Skolnick MH. 1994. A cell cycle regulator potentially involved in genesis of many tumor types. Science 264:436–440.

Kempster S, Phillips WA, Baindur-Hudson S, Thomas RJ, Dow C, Rockman SP. 2000. Methylation of exon 2 of p16 is associated with late stage oesophageal cancer. Cancer Lett 150:57–62.

Kumar R, Lundh RB, Louhelainen J, Hemminki K. 1998. Mutations in the CDKN2A (p16INK4a) gene in microdissected sporadic primary melanomas. Int J Cancer 75:193–198.

Li G, He L. 1986. A survey of the familial aggregation of esophageal in Yangcheng County, Shanxi Province. In: Wu M, Neberg DW, editors. Genes and disease. Proceedings of the First Sino-American Human Genetics Workshop. Beijing, China: Science Press. p 43–47.

Li JY. 1982. Epidemiology of esophageal cancer in China. Natl Cancer Inst Monogr 62:113–120.

Li MH, Li P, Li PJ. 1980. Recent progress in research on esophageal cancer in China. Adv Cancer Res 33:173–249.

Liggett WH, Jr., Sidransky D. 1998. Role of the p16 tumor suppressor gene in cancer. J Clin Oncol 16:1197–1206.

Liu Q, Yan YX, McClure M, Nakagawa H, Fujimura F, Rustgi AK. 1995. MTS-1 (CDKN2) tumor suppressor gene deletions are a frequent event in esophagus squamous cancer and pancreatic adenocarcinoma cell lines. Oncogene 10:619–622.

Merlo A, Herman JG, Mao L, Lee DJ, Gabrielson E, Burger PC, Baylin SB, Sidransky D. 1995. 5′ CpG island methylation is associated with transcriptional silencing of the tumour suppressor p16/CDKN2/MTS1 in human cancers. Nat Med 1:686–692.

Montesano R, Hollstein M, Hainaut P. 1996. Genetic alterations in esophageal cancer and their relevance to etiology and pathogenesis: a review. Int J Cancer 69:225–235.

Mori T, Miura K, Aoki T, Nishihira T, Mori S, Nakamura Y. 1994. Frequent somatic mutation of the MTS1/CDK4I (multiple tumor suppressor/cyclin-dependent kinase 4 inhibitor) gene in esophageal squamous cell carcinoma. Cancer Res 54:3396–3397.

Muzeau F, Flejou JF, Thomas G, Hamelin R. 1997. Loss of heterozygosity on chromosome 9 and p16 (MTS1, CDKN2) gene mutations in esophageal cancers. Int J Cancer 72:27–30.

National Cancer Control Office. 1980. Investigation of cancer mortality in China. Beijing, China: People's Health Publishing House.

Okamoto A, Demetrick DJ, Spillare EA, Hagiwara K, Hussain SP, Bennett WP, Forrester K, Gerwin B, Greenblatt MS, Serrano M. 1994. p16INK4 mutations and altered expression in human tumors and cell lines. Cold Spring Harb Symp Quant Biol 59:49–57.

Pollock PM, Welch J, Hayward NK. 2001. Evidence for three tumor suppressor loci on chromosome 9p involved in melanoma development. Cancer Res 61:1154–1161.

Randerson-Moor JA, Harland M, Williams S, Cuthbert-Heavens D, Sheridan E, Aveyard J, Sibley K, Whitaker L, Knowles M, Bishop JN, Bishop DT. 2001. A germline deletion of p14(ARF) but not CDKN2A in a melanoma-neural system tumour syndrome family. Hum Mol Genet 10:55–62.

Sherr CJ. 1996. Cancer cell cycles. Science 274:1672–1677.

Suzuki H, Zhou X, Yin J, Lei J, Jiang HY, Suzuki Y, Chan T, Hannon GJ, Mergner WJ, Abraham JM. 1995. Intragenic mutations of CDKN2B and CDKN2A in primary human esophageal cancers. Hum Mol Genet 4:1883–1887.

Tanaka H, Shimada Y, Imamura M, Shibagaki I, Ishizaki K. 1997. Multiple types of aberrations in the p16 (INK4a) and the p15(INK4b) genes in 30 esophageal squamous-cell-carcinoma cell lines. Int J Cancer 70:437–442.

Wu M, Hu N, Wang XQ. 1989. Genetic factor in the etiology of esophageal cancer and the strategy of its prevention in high-incidence areas of North China. In: HT Lynch and T Hirayama (eds), Genetic Epidemiology for Cancer. Boca Raton, FL: CRC Press, Inc. p 187–200.

Xing EP, Nie Y, Song Y, Yang GY, Cai YC, Wang LD, Yang CS. 1999a. Mechanisms of inactivation of p14ARF, p15INK4b, and p16INK4a genes in human esophageal squamous cell carcinoma. Clin Cancer Res 5:2704–2713.

Xing EP, Nie Y, Wang LD, Yang GY, Yang CS. 1999b. Aberrant methylation of p16INK4a and deletion of p15INK4b are frequent events in human esophageal cancer in Linxian, China. Carcinogenesis 20:77–84.

Zhou X, Tarmin L, Yin J, Jiang HY, Suzuki H, Rhyu MG, Abraham JM, Meltzer SJ. 1994. The MTS1 gene is frequently mutated in primary human esophageal tumors. Oncogene 9:3737–3741.